INTRODUCTION

Major histocompatibility complex class I (MHC-I) proteins display the cellular proteome, as small peptide fragments, at the cell surface to mediate T cell immune surveillance, which is essential for countering cellular abnormalities such as viral infections and cancer. The identification and characterization of antigen-specific T cells are of substantial importance for therapeutic development and mechanistic insights into immune-affected diseases. In 1996, Altman and colleagues (1) introduced the concept of fluorochrome-labeled MHC multimers to identify antigen-specific T cells. Since then, MHC multimer–based T cell detection approaches have evolved from the detection of few antigen-specific T cell populations per sample to the identification of more than 1000 specificities in parallel (2–6).

A major constraint that limits the flexibility, rapidity, and quality assurance of these state-of-the-art T cell detection platforms is the generation of large libraries of different peptide–MHC (pMHC) complexes, which often include 100 to 1000 different pMHC. To overcome this challenge, peptide exchange technologies have been developed to facilitate the generation of multiple pMHC from a single stock of a conditional ligand MHC-I molecule for a given human leukocyte antigen (HLA) or H-2 molecule. These strategies include the use of dipeptides as catalysts for peptide exchange (7), the widely used ultraviolet (UV)–mediated peptide exchange technology (8, 9), and a more recently developed temperature-induced exchange technology (10). Alternatively, individual small-scale parallel refolding reactions can be used (11); however, apart from requiring an additional peptide exchange process step, exchange technologies have disadvantages, including HLA–specific design and optimization, inefficient peptide exchange of low-affinity incoming peptides, the ability to multimerize only after peptide exchange, and time constraints related to duration of the exchange reaction.

We previously demonstrated that a disulfide-stabilized variant of the murine MHC-I molecule, H-2Kb, exhibits peptide-independent stability in the cellular context (12). A disulfide bond was introduced to link the α1 and α2 helices close to the F pocket of the peptide-binding groove to provide stability to the MHC-I complex. We further examined this molecule with the hypothesis that such disulfide-stabilized MHC-I molecules might be stable even in the absence of peptide and, hence, instantly peptide receptive. Such stable empty MHC-I molecules have the potential to become the ideal solution for rapid and flexible MHC multimer preparation. To achieve this, we introduced the same disulfide bond close to the F pocket of HLA-A*02:01 molecules. We show that these disulfide-stabilized HLA-A*02:01 molecules can be purified peptide-receptive and functionally empty after in vitro folding assisted by dipeptides (13). MHC-I multimers prepared with peptide-loaded disulfide-stabilized HLA-A*02:01, HLA-A*24:02, and H-2Kb molecules efficiently detect antigen–specific T cells. We further demonstrate the application of empty disulfide-stabilized MHC-I molecules to make peptide-loadable MHC-I tetramers that are advantageous for generating flexible reagents for T cell detection in highly antigen-variable situations.

RESULTS

Disulfide-stabilized empty MHC-I monomers

We designed a disulfide mutant variant of the human MHC-I protein HLA-A*02:01 (A2) by introducing two cysteines at positions 84 and 139 (replacing tyrosine and alanine, respectively), based on molecular dynamics (MD) simulations. MD simulations of this disulfide variant suggested that it had restricted conformational flexibility and a consequently increased stability of the peptide-free state compared with wild-type A2 (wt-A2), as measured by free energy profiles associated with the width of the peptide-binding cleft. Introducing the disulfide bond between the α1 and α2 helices at positions 84 and 139
elimated the tendency of the empty binding groove to collapse, suggesting that the disulfide variant would be stable even in the peptide-empty state while still holding the capacity to bind exogenous peptide (Fig. 1A and fig. S1). We then expressed the disulfide variant of A2 (hereafter called DS-A2) in *Escherichia coli*, isolated the denatured protein from inclusion bodies, and folded it in vitro with human β2-microglobulin (β2m). The folding reaction of DS-A2 was assisted by adding a small molecule with low affinity for MHC-I molecules, such as a dipeptide, which promotes the folding of MHC-I (13). Such dipeptides can be identified by a simple enzyme-linked immunosorbent assay (ELISA)–based screen for MHC-I folding efficiency (fig. S2). Folded DS-A2 molecules were purified by the standard size exclusion chromatography procedure, during which the small molecules dissociate from the MHC-I molecule, leaving a purified, functionally empty, and peptide-receptive molecule available for further use (Fig. 1B).

Functional evaluation of purified DS-A2 molecules demonstrated that the DS-A2 molecules were instantly peptide receptive and, hence, functionally empty, with a higher peptide on-rate (*K*on) than a dipeptide-loaded DS-A2 molecule, as shown by the binding of the fluorophore-labeled A2-restricted high-affinity peptide NLVPMVATV from cytomegalovirus (CMV) phosphoprotein 65 (pp65) to purified DS-A2 (Fig. 1C). We demonstrated, by thermal stability analysis, that the stability of empty DS-A2 molecules was greater than that of wt-A2 and that it further increased upon binding to a high-affinity peptide, suggesting the formation of a stable pMHC complex (Fig. 1D). Wt-A2 molecules that were folded in a similar process, using the dipeptide GM (glycyl-methionine), were likewise peptide receptive after folding with an on-rate (*K*on) comparable with the dipeptide-loaded DS-A2 molecule and displayed a similar thermal stability profile in the peptide-loaded state (fig. S3, A and B). The wt-A2 molecules fold with a low yield and are unstable in the purification process, whereas the DS-A2 can be efficiently purified with a high yield without dipeptide addition during the purification process (fig. S3, C and D). Thus, although we show no formal proof, all data are consistent with the assumption that the DS-A2 molecule is stable in an empty form, with no or minimal retention of the dipeptide in the binding groove. The stability and peptide-receptive profile of DS-A2 is accomplished by the disulfide bond close to the F pocket.

For further validation of the structural and functional properties of DS-A2, we generated a crystal structure with the NLVPMVATV peptide, which confirmed the formation of the disulfide bond between the α1 and α2 helices in the F pocket region of the peptide-binding groove (Fig. 1E). The additional disulfide bond did not alter conformation of the bound peptide or the A2 protein itself, as complete structural overlap was observed with the wt structure (Fig. 1, E and F).
In DS-A2, the C terminus of the peptide formed a new hydrogen bond with a water molecule because the hydroxyl group of tyrosine-84 was no longer available (Fig. 1G).

**T cell detection using peptide-loaded empty MHC-I molecules**

Because empty DS–MHC-I molecules are peptide receptive and can be instantly loaded with MHC-I peptide ligands, we next investigated whether pMHC-specific multimers prepared with this process can be used to detect antigen-specific CD8⁺ T cells (Fig. 2A). We loaded NLVPMVATV peptide onto empty DS-A2 molecules by direct incubation at room temperature for 15 min. The resulting pMHC monomers were multimerized on allophycocyanin (APC)–phycoerythrin (PE)–coupled streptavidin and used to stain A2-NLVPMVATV–specific CD8⁺ T cells from donor peripheral blood mononuclear cells (PBMCs). We compared the DS-A2 multimers with wt-A2 pMHC monomers prepared by UV-mediated peptide exchange (8). DS-A2 NLVPMVATV tetramers efficiently detected A2-NLVPMVATV–specific CD8⁺ T cells across different donors with a comparable frequency to the wt tetramers (Fig. 2B and fig. S4A). Unexpectedly, DS-A2 tetramers showed a higher fluorescence intensity and better separation from the tetramer-negative CD8⁺ T cell population than tetramers made from wt-A2, resulting in better staining (Fig. 2C).

We controlled that all DS and wt-A2 molecules were equally biotinylated (fig. S5). The folding efficiency of the DS molecules was superior to the wt (folded with UV ligand), but throughout all experiments, the molecular concentration of both molecules were identical (table S2). Further, in all cases, tetramer generation was conducted with an excess of pMHC monomer to ensure complete tetramerization and avoid biases from minor (nonmeasurable) variations in MHC monomer concentration.

Next, we tested the antigen-specific CD8⁺ T cell detection efficiency of DS-A2 tetramers with several known A2-restricted viral epitopes [Epstein-Barr virus (EBV) BRLF1 (YVLDHLIVV), CMV IE1 (VLEETSVML), and HIV polymerase (Pol) (ILKEPVHG)] in comparison with wt-A2 tetramers. The frequencies of all antigen-specific CD8⁺ T cells detected by DS-A2 tetramers were comparable with those detected by wt-A2 tetramers but with a consistently better staining index, even for very-low-frequency antigen-specific CD8⁺ T cells, suggesting that DS-A2 provides improved T cell staining irrespective of antigen specificity and the frequency of T cells (Fig. 2, C and D). No nonspecific T cell binding was observed when DS-A2 tetramers were associated with a nonbinding peptide or a negative control A2-binding peptide (ILKEPVHG from HIV Pol) (Fig. 2D).

To further investigate the potency of the DS-A2 in T cell detection, we compared DS-A2 with wt-A2, both classically folded with a panel of three virus-derived peptides or both folded with a UV-sensitive peptide followed by UV-activated ligand exchange (9). The resulting T cell staining was superior or equal when using DS-A2 compared with wt-A2 for all folding comparisons (Fig. 2E). In particular, the DS-A2 outperformed the UV exchange wt-A2 with all peptides tested, whereas for the classically folded molecules, no difference was observed (Fig. 2F).

To ascertain that the introduction of the disulfide bond does not alter the T cell receptor (TCR) recognition pattern of the pMHC complex, we determined the fingerprint of the TCR recognition using two T cell clones that both recognize a Merkel cell polyomavirus–derived epitope, A2-KLLEIAPNC, and carry different TCR sequences (14). We recently showed the use of DNA barcode–labeled MHC multimers for the in-depth characterization of the patterns that govern pMHC recognition (5, 15). This strategy provides a TCR fingerprint that identifies the requirements for the TCR–pMHC interaction. The TCR fingerprint was determined using 192 pMHC combinations derived from the original peptide KLLEIAPNC by the sequential replacement of each amino acid position with the 20 naturally occurring amino acids. Each of these peptide variants was either loaded onto DS-A2 empty monomers or exchanged for a UV-cleavable ligand in wt-A2. Then, MHC multimers were generated and tagged with DNA barcodes. A mixture of all pMHC multimer combinations was used to stain the T cell clones, allowing for a measure of the affinity-based TCR-pMHC interaction and consequently providing the TCR’s recognition preferences. Comparative analysis using both DS-A2 and wt-A2 molecules was applied to reveal potential differences in the TCR fingerprint. On the basis of the TCR fingerprints, no differences can be observed between the peptide recognition in the wt-A2 and DS-A2 variants (Fig. 2G).

Together, these data demonstrate that empty DS–MHC-I molecules can be used as an efficient tool for antigen-specific T cell detection. They eliminate the requirement for a peptide exchange process, and pMHC tetramers prepared using DS–MHC-I molecules improve the detection of antigen-specific CD8⁺ T cells across various viral antigenic specificities and frequencies compared with the UV exchange procedure.

**Application of empty MHC molecules in T cell detection strategies**

Next, we evaluated the use of empty DS–MHC-I molecules in pMHC multimer–based T cell detection strategies using combinatorial labeling of MHC-I multimers (3, 4). The pMHC specificities of eight well-characterized A2-restricted viral antigens were determined using empty DS-A2 monomers by direct incubation with peptide and, for comparison, with wt-A2 monomers loaded by UV-mediated peptide exchange. pMHC monomers were then tetramerized using streptavidin labeled with two different, predefined fluorochromes, forming a unique dual-color code for each individual pMHC. We analyzed healthy donor PBMCs by pooling all eight dual-color–coded pMHC tetramer specificities in a single staining. pMHC tetramers made from DS-A2 allowed the detection of several antigen-specific CD8⁺ T cell populations based on the dual-color code. We identified T cell populations specific to four of the eight tested viral antigens, and identical T cell populations, both in terms of specificity and frequency, were detected in parallel using wt-A2 pMHC tetramers (Fig. 3A). Again, an increased staining index was observed using DS-A2 pMHC tetramers (Fig. 3B), validating our observations from the single-color DS-A2 tetramers (Fig. 2, B to F). We further verified the antigen-specific T cell detection efficiency using DS-A2 pMHC tetramers in two additional donors (fig. S6).

We then extended the use of empty DS-A2 molecules to discover cancer-associated antigen–specific T cells in melanoma samples. Tumor-infiltrating lymphocytes (TILs) from melanoma lesions were expanded and analyzed for CD8⁺ T cell recognition against a library of 21 melanoma-associated antigens. In a single staining, using the combinatorial labeling approach, we discovered T cells specific to three cancer-associated antigens (gp100 ITDQVPFSV,
Fig. 2. Improved and efficient antigen-specific T cell detection using DS-A2 molecules. (A) Schematic illustration of pMHC multimer preparation using DS-A2. DS-A2 molecules were first incubated with the desired peptide for 15 min and then multimerized on fluorochrome-labeled streptavidin. (B) Dot plots of flow cytometry analysis for antigen-specific T cell detection across different donor samples. PBMCs were stained with DS-A2 and DS-A24 tetramers (DS) prepared after direct loading of peptides on DS monomers or with wt-A2 and wt-A24 tetramers (WT) prepared after UV-mediated peptide exchange on wt monomers. (C) Staining index (SI) of DS (blue) and WT (orange) tetramers for plots in (B) (and one additional donor) and (D). Paired t test P = 0.0004 (**). The staining index was calculated as (median of positive − median of negative)/(SD of negative × 2). (D) Dot plots of T cells stained by DS-A2 (DS) and wt-A2 (WT) tetramers for several virus-associated antigens in one donor’s PBMCs. As controls, an HIV-derived A2 epitope and a nonbinding peptide were used. (E) Representative dot plots of FLU MP (GILGFVFTL)–specific T cell detections in donor PBMCs comparing DS-A2 and wt-A2 tetramers assembled from pMHC monomers produced by classical folding, UV-mediated peptide exchange or using empty DS-A2. (F) Staining index comparison for tetramers prepared using DS- and wt-A2 molecules across several viral specificities and donor PBMCs. Paired t test was used for statistical analysis; P = 0.015 [*] (DS-UV versus WT-UV) and P = 0.013 [*] (WT-UV versus empty DS). ns, not significant. (G) TCR recognition profile of DS-A2 and wt-A2 molecules. Shannon logos display the amino acid requirements for pMHC-TCR interaction at each position of the peptide. TCR recognition profiles were generated using a DNA barcode–labeled library of 192 peptide-A2 multimers with amino acid substitutions of the original KLLEIAPNC peptide. TCR recognition for each of these peptide variations was measured with pMHC dextramers prepared from DS-A2 molecules or from wt-A2 molecules for two T cell clones that recognized the Merkel cell polyomavirus–derived epitope A2-KLLEIAPNC. Numbers on dot plots represent the percentage of pMHC multimer–positive cells out of total CD8+ T cells.

gp100 AMLGTHMEV, and Mart-1 EAAGIGILTV; Fig. 3C). Again, both the specificity and the frequency of antigen-specific CD8+ T cells were comparable with the wt-pMHC tetramers, but an increased staining index was observed when using the DS-A2 (Fig. 3D). The Mart-1 EAAGIGILTV cancer antigen is a known low-affinity A2 peptide, and detection of Mart-1–specific T cells using DS-A2 further establishes that the disulfide-stabilized molecule can be loaded even with a very low-affinity peptide while still obtaining a functional
interaction with T cells. Across all samples, including detection of both virus-derived and cancer-associated antigen-specific T cells, we demonstrate a tight correlation in the observed T cell frequency, using either wt- or DS-A2 for the T cells (Fig. 3E).

To further validate the capacity for T cell detection in a situation when T cells are expected to have low TCR-pMHC avidity, we made the tetramers using either wt- or DS-A2 for the T cells (Fig. 3E). Moreover, for the wt-A2 tetramer, we observed a substantial loss of staining intensity starting after 12-hour incubation with wt-A2 (Fig. 4E). Moreover, for the wt-A2 tetramer, we observed a substantial loss of staining intensity starting after 12-hour incubation with wt-A2 tetramer, whereas DS-A2 tetramer–stained T cell population over that of wt-A2 tetramer stayed intact (Fig. 4E). Furthermore, similar to the wt-A2 tetramer, DS-A2 tetramer also retained their full staining capacity after 7 days (Fig. 4D and fig. S8).

Second, we compared the stability of DS-A2 tetramers with wt-A2 tetramers from donor PBMCs and in the patient with melanoma [combined from Figs. 2 (B, D, and E) and 3 (A and C) and fig. S6]. (F) Comparative staining index and frequency of OT1 splenocytes detected by DS and wt tetramers of murine MHC H-2Kb. SIINFEKL peptide and six variant peptides, highlighted in red, with different affinities to the OT1-TCR (plotted high to low on the x axis) were used to compare the detection efficiency of DS with wt–H-2Kb tetramers. Dot plots are shown in fig. S7.

Empty-loadable tetramers for neoantigen discovery

To further ease the pMHC tetramer production, we explored the use of empty prelabeled MHC tetramers for postassembly loading with the peptide of interest as a simple and flexible approach to MHC multimer–based T cell detection. Empty-loadable tetramers can be converted to pMHC-specific tetramers by direct incubation with the desired peptide (Fig. 4A). pMHC tetramers are generated instantly after addition of peptide (1 min), but increased peptide-loading time can be used for low-to-medium affinity peptides (Fig. 4B). We prepared DS–empty-loadable tetramers (DS-ELTs) for A2, A24, and H-2Kb. All three MHC-I allotypes formed DS-ELTs that were suitable for T cell detection (Fig. 4C).

We further evaluated the functional stability of DS-ELTs both before and after peptide loading. First, we kept DS-ELTs at 4°, 20°, or 37°C for 0 to 168 hours before peptide loading to determine their stability in the empty form. Both at 4° and 20°C, the DS-ELTs retained their full staining capacity after 7 days (Fig. 4D and fig. S8). Second, we compared the stability of DS-A2 tetramers with wt-A2 tetramers after peptide loading of GILGFVFTL. The tetramers were filtered to remove excess peptide and incubated at the indicated temperatures (4°, 20°, and 37°C) for 0 to 168 hours. The results again showed an increased mean fluorescence intensity (MFI) of the GILGFVFTL DS-A2 tetramer–stained T cell population over that of wt-A2 (Fig. 4E). Moreover, for the wt-A2 tetramer, we observed a substantial loss of staining intensity starting after 12-hour incubation with wt-A2 tetramer, whereas DS-A2 tetramer–stained T cell population over that of wt-A2 tetramer stayed intact (Fig. 4E).
Empty-loadable tetramers

Fig. 4. Empty-loadable MHC-I multimers for efficient detection of antigen-specific T cells. (A) Schematic illustration of empty-loadable MHC-I multimers assembled using DS–MHC-I molecules. Empty-loadable DS-A2 multimers were stored at −20°C and loaded with peptide on demand. (B) Conditions for peptide loading to empty-loadable multimers. Detection of GILGFVFTL-specific T cells after addition of FLU MP GILGFVFTL peptide is shown as the frequency of multimer-positive CD8+ T cells at varying peptide concentration and time. (C) Detection of antigen-specific CD8+ T cells using DS-ELTs for A2, A24, and H-2Kb MHC alleles compared with wt and DS tetramers. Wt tetramers were prepared after UV-mediated peptide exchange. (D) Stability of empty-loadable multimers. DS-A2 ELTs conjugated with APC were stored for up to 1 week at 4°C, 20°C, and 37°C and then loaded with CMV pp65 (NLVPMVATV)–specific peptide to detect T cells from donor PBMCs. The frequency (left) and MFI (right) of detected antigen-specific T cells are shown for varying conditions. Dot plots are shown in fig. S8. (E) Peptide dissociation rate measured as FLU MP GILGFVFTL–specific T cell detection efficiency by wt-A2 (orange) or empty-loadable DS-A2 multimers (blue). pMHC multimers (without excess peptide) were incubated at 4°C, 20°C, and 37°C up to 1 week and used for T cell detection from donor PBMCs. The frequency (left) and MFI (right) of detected antigen-specific T cells are shown for varying conditions. Dot plots are shown in fig. S9. (F) Dot plots showing T cell detection efficiency of DS-A2 ELTs stored for 6 months. DS-A2 ELTs conjugated with different fluorophores stored at −20°C for 6 months were incubated with FLU MP peptide and used for T cell detection. Compare with freshly prepared DS-A2 ELTs in (C).
Detection of T cell recognition to cancer neoantigens

In cancer immunotherapy, there is considerable interest in understanding T cell recognition of mutation-derived neoepitopes and the potential to exploit these for therapeutic strategies. In most cases, large pMHC library screening is necessary for a comprehensive evaluation of neoepitope recognition (16, 17). The ability to apply post-loadable MHC multimers of DS-A2 may ease the process of generating such large pMHC libraries.

To prove the value of DS-A2 for the detection of neoantigen-responsive T cells in patients with cancer, we analyzed TILs from a patient with melanoma for the recognition of mutation-derived neoepitopes. On the basis of tumor DNA whole-exome and RNA sequencing, 276 mutations were identified. From these mutations, 43 A2-restricted neoepitopes were predicted using MuPeXi (18) with an A2 binding rank score of <2 for the mutant peptide variant (using netMHCpan v2.8). We used empty-loadable tetramers to detect neoantigen-specific T cells in the TIL sample. A library of 54 peptides, including 48 cancer-derived peptides (43 from neoantigens and 5 from melanoma-associated antigens) and 6 virus-derived epitopes, were analyzed for T cell recognition using empty DS-A2 tetramers post-loaded with peptide. Using combinatorial fluorescence labeling, we identified CD8+ T cells specific to eight of the predicted neoantigens; several of these neoantigens shared overlapping peptide sequences (Fig. 5, A and B). We also identified T cells for three melanoma-associated antigens (gp100 AMQLTHMETV, Mart-1 EAAAGILTIV, and gp100 ITDQVPFSV), which were previously detected in a different TIL preparation from this patient (19), and for two viral antigens (EBV BRLFI, YVLDHLIVV; FLU MP, GILGFVFTL) (Fig. 5, A and B). Together, we demonstrate here the successful application of empty-loadable tetramers in the discovery of cancer-associated antigens, including mutation-derived neoepitopes.

DISCUSSION

We here introduce empty-loadable MHC-I multimers as a tool for improved T cell detection. We demonstrate their potential in the identification of T cells specific to cancer-associated antigens, including mutation-derived neoantigens (Fig. 5). These stable, ready-to-use MHC-I multimers, which can be converted to antigen-specific pMHC multimers in a single-step peptide addition, perfectly align with the state-of-the-art antigen-specific T cell detection platforms that require the rapid generation of large arrays of MHC multimers (6, 20). We have shown the feasibility of using DS-A2 in both combinatorial color encoding strategies and DNA barcoding–based methods of T cell detection (Figs. 2, 3, and 5). Empty-loadable MHC-I multimers will be advantageous for the fast and effective evaluation of T cell recognition for personalized immunotherapy (21).

The disulfide bond–stabilized MHC-I molecules do not require MHC-restricted high-affinity peptide either for their in vitro folding or for stability after folding (as required for wt–MHC-I molecules), and they remain peptide receptive even after long-term storage at −20°C. Thus, they provide a flexible and convenient peptide-loading system that does not require the use of peptide exchange technologies. Furthermore, antigen-specific A2 tetramers prepared with empty DS-A2 provide better T cell staining (as measured by the staining index) than wt-A2 multimers of the same antigen specificities prepared via UV-mediated peptide exchange technology (Figs. 2 to 4) (8, 9). A possible explanation for the increased staining index using DS-MHC relates to the potentially incomplete removal of photo-cleavable peptide or the retention of partly unfolded empty wt-A2 molecules after peptide exchange (8) that may result in fewer functional molecules per tetramer, potentially reducing the functional tetrameric form to a trimeric form. This is supported by the minimal differences observed between conventionally folded pMHC-I complexes of either wt or DS form (Fig. 2). Another contributing factor is the enhanced stability of peptide-loaded DS-A2 molecules, compared with wt-A2 molecules, which may affect the staining capacity (Fig. 4).

The specific locations for the insertion of the two cysteines forming the disulfide bond (Y84C and Y139A) is conserved across MHC-I molecules. Hence, we expect this strategy to be applicable across all MHC-I allotypes. For each allotype, a suitable short peptide (d- or tripeptide) will need to be identified via screening to facilitate the folding reaction. Here, we demonstrate the use of DS versions of A2, A24, and H-2Kb, as three examples of MHC-I molecules frequently applied for immunological investigation.

Empty-loadable MHC multimers provide a fast and convenient option to generate antigen-specific MHC multimers, which has advantages over other methods. The UV exchange strategy requires a longer peptide exchange period and is inefficient for peptide exchange in pretetramerized reagents (9). An alternative technology using peptide exchange on MHC multimers was recently pursued on the basis of temperature-mediated peptide exchange that uses a HLA-specific, low-affinity peptide to exchange for the incoming peptide directly on MHC multimers at an elevated temperature (10); however, in the temperature exchange system, an incoming peptide competes with a low-affinity, temperature-sensitive ligand, which may compromise the exchange for low-affinity incoming peptides, and A2 multimers require substantially more time for peptide exchange to be completed (3 hours). Similarly, all peptide exchange technologies require the design of HLA-specific ligands (photo-cleavable peptides or peptides suitable for temperature exchange) for optimal peptide exchange processes, whereas the generation of empty DS-MHC monomers is free of such requirements. DS-MHC molecules require the assistance of small molecules, such as dipeptides, for their folding, but these are relatively cheap and easy to identify (fig. S2) (13).

An important concern relates to the induction of potential changes in peptide recognition when peptide is presented in the DS-A2 versus wt-A2. Empty DS–MHC-I molecules are a non-natural variant, due to the insertion of the additional disulfide bond. Although it is formed outside the peptide-binding area, the extra disulfide bond might have functional implications either on peptide binding or on T cell recognition. To address this, we obtained the crystal structure of the CMV pp65 NLVPMVATV/DS-A2 complex and determined the
Neoantigens

Cancer antigens
Viral antigens

Fig. 5. Detection of neoantigen-specific T cell recognition in cancer. (A) Antigen-specific T cells in melanoma TILs identified by the combinatorial color encoding strategy using empty-loadable DS-A2 multimers. pMHC multimers for 54 A2-restricted peptides (43 neoantigens, 5 melanoma-associated cancer antigens, and 6 virus-derived antigens) were generated, each in a two-color combination. Frequency of pMHC multimer–positive cells out of total CD8+ T cells is shown as colored dots across three categories. Multimer-negative cells are plotted in gray for each pMHC combination (zero values are converted to 0.0001 to plot on log scale). (B) Dot plots of the antigen-specific T cell populations from (A), each is shown with indicated fluorochromes and peptide sequences. The mutated amino acid position of the neoantigens is highlighted in red.

TCR fingerprint of two T cell clones, using either the DS-A2 or the wt-A2. The crystal structure demonstrates identical peptide binding properties of wt versus DS-A2 (Fig. 1). The TCR fingerprint likewise shows identical recognition of the DS–pMHC-I complex compared with the wt (Fig. 2). DS-A2 empty MHC multimers are functional after long-term storage, and pMHC-specific conversion of them is independent of any exchange technology that may alter their quality. Hence, they provide an efficient tool to produce quality-assured reagents for immune monitoring, for diagnostics, and in therapeutic applications such as the isolation or specific stimulation of antigen-specific T cells for adaptive T cell transfer in immunotherapy approaches (21, 22).

MATERIALS AND METHODS

Study design
This study was designed to investigate the use of empty DS–MHC-I molecules as an advanced tool for the detection of human CD8+ T cells using antigen-specific pMHC multimers and to explore the advantages of DS–MHC-I molecules for identification of cancer-associated antigens, including neoantigens, over the existing methods. We used established technologies for T cell detection to demonstrate the applicability of DS–MHC-I molecules. The DS–MHC-I molecules were designed on the basis of MD simulation analysis and previously available literature. Standard biochemical assays and crystal structure analysis were performed to validate the peptide-receptive empty MHC-I molecules. We illustrated the use of DS-MHC multimers across two human MHC alleles (A2 and A24) and one murine MHC allele (H-2Kb). For identification of antigen-specific T cells, PBMCs from healthy volunteers, splenocytes from OT1 and OT3 transgenic mice, and TILs from a patient with melanoma were used.

Production of MHC-I heavy chains and β2m
MHC-I heavy chains and β2m were produced in E. coli, as described previously (23, 24). Briefly, proteins were expressed in E. coli strain BL21(DE3)pLysS using pET series plasmids. Inclusion bodies containing expressed proteins were harvested using sonication in lysis buffer followed by washing in detergent buffer and wash buffer and solubilizing the protein in 8 M urea buffer [8 M urea, 50 mM K·Hepes (pH 6.5), and 100 μM β-mercaptoethanol]. Proteins were stored at −80°C until they were used for in vitro folding.

In vitro folding and purification of MHC-I molecules
MHC-I heavy chain (1 μM) and β2m (2 μM) were diluted in a folding buffer [0.1 M tris (pH 8.0), 500 mM l-arginine–HCl, 2 mM EDTA, 0.5 mM oxidized glutathione, and 5 mM reduced glutathione] with 60 μM high-affinity peptide (NLVMPYATV, GILGFVFITL, or YVLDDLIVVV) or photocleavable peptide (KILGFVF-J-V;DS and wt-A2, VYG-J-VRACL; wt-A24, FAPGNY-J-AL; wt–H-2Kb) or with
10 mM dipeptide GM (DS and wt-A2), glycyl-leucine (GL) (DS–H-2K\(^{\beta}\)) (13), or glycyl-phenylalanine (GP) (DS–A24) and incubated at 4°C for 3 to 5 days, followed by concentrating folded proteins with 30-kDa cutoff membrane filters (Vivaflo 200; Sartorius). Proteins were then biotinylated with the BirA biotin-protein ligase standard reaction kit (Avidity LLC, Aurora, CO). Biotinylated MHC-I monomers were purified by size exclusion chromatography using high-performance liquid chromatography (Waters Corporation, USA) or an ÄKTA HiLoad 26/600 Superdex 200 gel filtration column (bed volume, 120 or 320 ml) or Superdex 200 10/300 GL (bed volume, 24 ml) (GE Healthcare Life Sciences). All MHC-I-folded monomers were quality-controlled for their concentration, biotinylation efficiency, and UV degradation (wherever applicable) and stored at −80°C until further use. For DS–A2 folded with dipeptide GM, two different batches of purified protein were used for the data shown in Figs. 2 and 4.

**Electrophoretic mobility shift assay**

After biotinylation, purified MHC-I monomers were tested for biotinylation efficiency. Purified monomers (2 μg) were incubated with 4 μg of Streptavidin-plus (ProZyme SA10) for 30 min at 4°C. After incubation, proteins were separated on 4 to 20% Mini-PROTEAN TGX Gel (Bio-Rad, 4561095) along with a PAGE ruler prestained protein ladder (Bio-Rad, 1610374) and stained with Coomassie (Bio-Rad, 161-0786).

**In vitro folding of MHC-I molecules for dipeptide screening**

In vitro folding of MHC-I molecules was performed in black, nonbinding, 96-well plates in 250-μl volume. Heavy chain and βm were used at the concentration of 3 μM (100 μg/ml) and 8 μM (100 μg/ml), respectively, in folding buffer [0.1 M tris (pH 8.0), 500 mM L-arginine–HCl, 2 mM EDTA, 0.5 mM oxidized glutathione, and 5 mM reduced glutathione]. Dipeptide (20 mM) was maintained throughout the sample wells, whereas 10 μM high-affinity HLA-specific peptides (DS–A2, NLVPMVATV; DS–A24, QYDPVAALF) were used for positive control wells. The folding reactions were then incubated at 4°C and 300 rpm (plate shaker) for 7 days. After folding, the plates were centrifuged at 3000g for 15 min, and samples were diluted 50-fold for competitive ELISA assay.

**Competitive ELISA assay**

For competitive ELISA assay, clear, high-binding, 96-well microplates (3590, Corning) were coated overnight with 100 μl per well of A2-NLVPVATV (10 μg/ml) prepared in carbonate coating buffer [15 mM Na\(_2\)CO\(_3\) and 35 mM NaHCO\(_3\) (pH 9.5)]. Plates were washed (2 × 300 μl) in wash buffer [TWEEN 20 and 50 mM tris-HCl (pH 8.0)] and blocked with 150 μl of blocking buffer [1% bovine serum albumin (BSA) and 50 mM tris-HCl (pH 8.0)] at 4°C for 2 hours. Samples or standards (folded MHC-I molecules, e.g., A2-NLVPVATV complex) were diluted in dilution buffer [1% BSA, 0.05% TWEEN 20, and 50 mM tris-HCl (pH 8.0)]. Diluted samples were preincubated in a clear, U-bottom, nonbinding 96-well plate (650901, Greiner) with W6/32 antibody (15 ng/ml) in dilution buffer for 1 hour, after which 100 μl of this mixture was transferred to the blocked assay plate after two washes in wash buffer and incubated at 4°C for 2 hours. After incubation, wells were washed twice with wash buffer, and 100 μl of horseradish peroxidase–conjugated goat anti-mouse immunoglobulin G (115-035-003, Jackson ImmunoResearch Europe Ltd.) was added (1:2000 dilution in dilution buffer) and incubated for 1 hour at room temperature. After another five washes, 100 μl of TMB substrate (34021, Thermo Fisher Scientific) was added to each well and incubated at room temperature for 10 min. Reaction was immediately stopped by adding equal volume of 2 M H\(_2\)SO\(_4\) solution. Fluorescence intensity was measured at 450-nm absorbance in a Tecan Infinite M1000 PRO (Tecan Group Ltd., Switzerland) plate reader.

*Standard curve was plotted using GraphPad Prism software, and concentrations for folded MHC-I molecules in the unknown samples were interpolated. Folding efficiencies were calculated relative to the folding with the high-affinity full-length peptide.

**Peptide binding measured by fluorescence anisotropy**

To assess peptide binding by fluorescence anisotropy, 100 nM fluorochrome-labeled peptide NLVPK\(_{FITC}\) VATV (from GeneCust Biotechnology) was added to 300 nM purified folded DS–A2 MHC-I complexes ± GM, and kinetic measurements were performed with a Tecan Infinite M1000 PRO (Tecan, Crailsheim, Germany) multimode plate reader by measuring anisotropy [fluorescein isothiocyanate (FITC) \(\lambda_{ex} = 494\) nm, \(\lambda_{em} = 517\) nm]. For peptide binding studies, wt-A2 molecules folded with dipeptide were purified with the size exclusion chromatography buffer containing 10 mM GM. All peptide binding measurements were performed at room temperature (22° to 24°C) in 50 mM Hepes buffer (pH 7.5) with a total reaction volume of 100 μl. Data were plotted using GraphPad Prism software.

**Thermal stability measurements**

Thermal stability was measured using differential scanning fluorimetry by dissolving MHC-I molecules in 50 mM Hepes buffer (pH 7.5) at 200 μg/ml. Experiments were carried out using the Prometheus NT.48 (NanoTemper Technologies, Germany). Each of the capillaries was loaded with 10 μl of test samples in either duplicates or triplicates. The temperature gradient was set to an increase of 1°C/min in a range of 20° to 80°C. Protein unfolding was measured by detecting the temperature-dependent change in tryptophan fluorescence at emission wavelengths of 330 and 350 nm. Thermal stability of purified empty DS–A2 molecules was measured alone or in the presence of 10 mM dipeptide GM or after incubating with 10 μM NLVPVATV peptide. Melting curve and \(T_m\) (melting transition temperature) values were generated by the PR.ThermControl v2.1 software from the first derivative of the fluorescence at 350 nm and plotted using GraphPad Prism software.

**MD simulations**

For all simulations, the Amber16 Molecular Dynamics Package in combination with the parm14SB force field for proteins was used (25). The Protein Data Bank (PDB) 3GSO structure of A2 in complex with the peptide NLVPVATV served as the start structure for the wt–A2. For the DS–A2, the reported structure served as the start structure. The same start structures without coordinates for the bound peptide served as start structures for simulations in the absence of bound peptide. The structures were solvated in octahedral TIP3P (transferable intermolecular potential with 3 points) water (26) boxes, keeping a 10-Å minimum distance between the structures and the box boundaries. Sodium and chloride ions were added to achieve neutrality and a salt concentration of 0.15 M. After extensive energy minimization (2500 steps), the systems were heated to 300 K (within 50 ps), keeping the solute (heavy atoms) restraint to the starting positions (force constant, 25 kcal mol\(^{-1}\) Å\(^{-2}\)), followed by gradual removal of the positional restraints over a period of 0.5 ns. After
equilibration, the unrestraint data gathering MD simulations were extended up to 1 μs, and coordinates were saved every 2 ps. The hydrogen mass repartitioning scheme was used, allowing a 4-fs time step (27). Analysis of trajectories was performed using the cpptraj module of Amber16. The distance between the centers of mass of residues 66 to 84 in the α1 helix and residues 139 to 159 in the α2 helix of MHC-I molecules was used to monitor the width of the peptide-binding cleft. Free energy profiles were calculated on the basis of the center-of-mass distance probability distribution observed during the unrestraint MD production simulations. Relative free energies were obtained from the distance probability distribution \( p(d) \) using the equation \( dG = -RT \ln(p(d)) \), with \( R \) indicating the gas constant and \( T \) indicating the temperature (300 K).

**Crystal structure determination**

The DS-A2 mutant in complex with the NLVPMVATV peptide was concentrated to 10 mg/ml, and crystals were obtained using a mother liquor containing 5 mM cobalt chloride, 5 mM cadmium chloride, 5 mM magnesium chloride, 5 mM nickel chloride, 0.1 M Hepes (pH 7.5), and 12% polyethylene glycol (PEG) 3350. A single crystal was transferred to a cryo-protectant solution containing 5 mM cobalt chloride, 0.1 M Hepes buffer (pH 7.5), 15% PEG 3350, and 10% glycerol. The crystal was mounted and cryo-cooled to 100 K on the EMBL P13 beamline at HESY (Deutsches Elektronen-Synchrotron, Hamburg, Germany) containing an EIGER × 16M detector. An x-ray data set was collected at a wavelength of 0.976 Å to a resolution of 1.5 Å (table S1). The data were processed with XDS (28) and scaled with AIMLESS (29). Molecular replacement was performed using MOLREP (30) with the coordinates of an A2 molecule (PDB ID, 3GSO), and the structure was refined with REFMAC5 (31). The engineered disulfide bond was manually built with Coot (32). The refined coordinates of an A2 molecule (PDB ID, 3GSO), and the structure was refined with REFMAC5 (31) with \( R \) factor of 17.5% (\( R_{	ext{free}} \) 20.0%). MolProbity (33) was used to validate the geometry and indicated that 98.7% of the residues were in the allowed regions of the Ramachandran plot (with 1.3% of the residues in the generously allowed regions and no residues in the disallowed regions).

**Preparation of MHC multimers**

To prepare pMHC multimers using wt-A2, DS-A2, wt-A24, and wt-H-2Kb monomers, pMHC complexes with desired peptide were generated by UV-mediated peptide exchange technology (8). Briefly, purified UV light–sensitive pMHC complex (100 μg/ml) (A2, KILGPVFJ-V; A24, VVG-J-VRACL; H-2Kb, FAPGNY-J-AL; J represents UV cleavable moiety) was incubated with 200 μM peptide in phosphate-buffered saline (PBS) for 1 hour under UV light (366 nm). pMHC complexes of DS-A2, DS-A24, and DS-H-2Kb were prepared by incubating purified DS-MHC monomers (100 μg/ml) with 100 μM peptide for 15 min in PBS. Classicly folded A2 pMHC monomers were directly used for multimer preparation. pMHC complexes were centrifuged for 5 min at 10,000g, and supernatants were used to make MHC multimers. For each 100 μl of pMHC monomers, 9.02 μl [0.2 mg/ml stock, SA-PE (BioLegend, 405204), SA-PE-Cy7 (streptavidin-PE/Cy7, BioLegend, 405206), and SA-APC (BioLegend, 405207)] or 18.04 μl [0.1 mg/ml stock, SA-BUV395 (Brilliant Ultraviolet 395; BD 564176), SA-BV421 (Brilliant Violet 421; BD 563259), SA-BV605 (Brilliant Violet 605; BD 563260), SA-BV650 (Brilliant Violet 650; BD 563855), and SA-BUV737 (Brilliant Ultraviolet 737; BD 564293)] of streptavidin conjugates was added and incubated for 30 min at 4°C, followed by addition of d-biotin (Sigma-Aldrich) at a final concentration of 25 μM to block any free binding site. Empty-loadable MHC multimers were prepared from DS-A2 monomers by incubating 100 μl of monomers with 9.02 μl (0.2 mg/ml stock) or 18.04 μl (0.1 mg/ml stock) of streptavidin conjugates for 30 min at 4°C and stored at −20°C with 5% glycerol and 0.5% BSA. Empty-loadable MHC multimers were converted to pMHC multimers by incubating 100 μM peptide (unless stated otherwise) for 15 min at 4°C. For combinatorial encoding strategy (34), pMHC multimers of each peptide specificity were prepared in two colors after any of the above processes and mixed in a 1:1 ratio before staining the cells.

**T cell staining and analysis**

For human T cell staining, pooled pMHC multimers (in combinatorial encoding) or pMHC multimers of individual specificity were incubated with 1 × 10^6 to 2 × 10^6 PBMCs or expanded TILs from a melanoma lesion [thawed and washed twice in RPMI 1640 +10% fetal calf serum (FCS) and washed once in fluorescence-activated cell sorting (FACS) buffer; PBS + 2% FCS] for 15 min at 37°C in 80-μl total volume. Cells were then mixed with 20 μl of antibody staining solution CD8-BV480 (BD B566121) or CD8-BV510 (BD 563919) (final dilution, 1:50), dump channel antibodies CD4-FITC (final dilution, 1:80; BD 345768), CD14-FITC (final dilution, 1:32; BD 345784), CD19-FITC (final dilution, 1:16; BD 345776), CD40-FITC (final dilution, 1:40; Serotec, MCA1590F), and CD16-FITC (final dilution, 1:64; BD 335035), and a dead cell marker (final dilution, 1:1000; LIVE/DEAD Fixable Near-IR; Invitrogen, L10119) and incubated for 30 min at 4°C. Cells were then washed twice in FACS buffer (PBS + 2% FCS) and acquired on a flow cytometer (LSRFortessa, Becton Dickinson). Lymphocytes were gated on the basis of forward and side scatter, and multimer-positive CD8 T cells were identified from singlets and live lymphocytes according to the above detailed cell surface markers (fig. S4) (3,35). For a detailed comparison between wt- and DS-MHC multimers, we have chosen to illustrate MHC multimer–positive CD8 T cells on singlets and live lymphocytes to elucidate any DS-pMHC multimer–specific binding to CD8^+ population (Fig. 2 and fig. S4). However, for routine T cell identification, a more stringent gating strategy is recommended to exclude any non–CD8 T cell–associated multimer binding. Data were analyzed and plotted using FACSDivia (Becton Dickinson) or FlowJo analysis software. For staining for mouse splenocytes, OT1 and OT3 T cells, the following antibodies were used: CD8α-BV480 (BD 566096), CD3-FITC (BioLegend, 100206), and dead cell marker (LIVE/DEAD Fixable Near-IR; Invitrogen, L10119). MHC multimer–positive cells were identified using the gating strategy illustrated in fig. S4B.

**TCR fingerprinting analysis**

For the TCR fingerprinting analysis, DNA barcode–labeled pMHC multimers were generated as described previously (5) using PE-labeled streptavidin-conjugated dextran obtained from Fina Biosolutions (Rockville, USA). PE-labeled dextramers were incubated with pMHC monomers (192 pMHC combinations derived from the original peptide KLLEIAPNC by sequential replacement of each amino acid position by the 20 naturally occurring amino acids, prepared using either wt-A2 or DS-A2) for 30 min. Resulting pMHC multimers were used to stain A2-KLLEIAPNC–specific T cell clones (2 × 10^6 cells). After staining, cells were acquired on FACSaria Fusion (Becton Dickinson). PE-positive CD8^+ T cells from each sample were sorted and subjected to polymerase chain reaction (PCR) amplification of their associated DNA barcodes. PCR-amplified DNA barcodes were then mixed with 20 μl of antibody staining solution CD8-BV480 (BD B566121) or CD8-BV510 (BD 563919) (final dilution, 1:50), dump channel antibodies CD4-FITC (final dilution, 1:80; BD 345768), CD14-FITC (final dilution, 1:32; BD 345784), CD19-FITC (final dilution, 1:16; BD 345776), CD40-FITC (final dilution, 1:40; Serotec, MCA1590F), and CD16-FITC (final dilution, 1:64; BD 335035), and a dead cell marker (final dilution, 1:1000; LIVE/DEAD Fixable Near-IR; Invitrogen, L10119) and incubated for 30 min at 4°C. Cells were then washed twice in FACS buffer (PBS + 2% FCS) and acquired on a flow cytometer (LSRFortessa, Becton Dickinson). Lymphocytes were gated on the basis of forward and side scatter, and multimer-positive CD8 T cells were identified from singlets and live lymphocytes according to the above detailed cell surface markers (fig. S4) (3,35). For a detailed comparison between wt- and DS-MHC multimers, we have chosen to illustrate MHC multimer–positive CD8 T cells on singlets and live lymphocytes to elucidate any DS-pMHC multimer–specific binding to CD8^+ population (Fig. 2 and fig. S4). However, for routine T cell identification, a more stringent gating strategy is recommended to exclude any non–CD8 T cell–associated multimer binding. Data were analyzed and plotted using FACSDivia (Becton Dickinson) or FlowJo analysis software. For staining for mouse splenocytes, OT1 and OT3 T cells, the following antibodies were used: CD8α-BV480 (BD 566096), CD3-FITC (BioLegend, 100206), and dead cell marker (LIVE/DEAD Fixable Near-IR; Invitrogen, L10119). MHC multimer–positive cells were identified using the gating strategy illustrated in fig. S4B.
were purified with the QIAquick PCR Purification kit (Qiagen, 28104) and sequenced at SeqeuTtech (USA) or GeneDx (USA) using Ion Torrent PGM 314 or 316 chip (Life Technologies). Sequenced barcodes were then analyzed using Barracoda (www.cbs.dtu.dk/services/barracoda) to identify the log2 fold change of the barcode signal related to the sorted cell population versus the signal of the barcode in the total MHC multimer library before T cell staining. The TCR fingerprints were created on the basis of the log2 fold changes calculated using Barracoda. The amino acid substitution changes calculated using Barracoda. The amino acid substitution changes were then analyzed using Barracoda (www.cbs.dtu.dk/services/barracoda) to identify the log2 fold change of the barcode sequence. The log2 fold change of the original peptide was assigned to each of the respective amino acid residues at each position. In this way, a position-specific scoring matrix (PSSM) for each clone was calculated with the number of rows corresponding to the number of positions in the peptide and the number of columns corresponding to the number of naturally occurring amino acids. We then normalized each row of the PSSMs to sum to 1, and the resulting position-specific frequency matrices were then converted into Shannon logos.

**Ethical approval**

All healthy donor and patient materials were collected under approval by the local Scientific Ethics Committee, and written informed consent was obtained according to the Declaration of Helsinki. Healthy donor PBMCs were obtained from the central blood bank, Rigshospitalet, Copenhagen, in an anonymized form. TILs from patients with melanoma were obtained on the basis of collaboration with Herlev Hospital, Department of Oncology and the National Center for Cancer Immune Therapy, and approved by the local ethics committee. Splenocytes from OT1 and OT3 transgenic mice were collected by collaborators, under regular approval from the national committee of animal health (approval no. M165-15, University of Lund, Sweden).

**Lymphocyte preparations**

PBMCs from healthy donors were isolated from buffy coat blood products by density centrifugation on Lymphoprep (Axis-Shield PoC) and cryopreserved at −150°C in FCS (Gibco) + 10% dimethyl sulfoxide (DMSO). TILs were obtained from resected tumor lesions from patients with stage IV melanoma (American Joint Committee on Cancer). Tumor lesions were resected after surgical removal. Tumor fragments were cultured individually in complete medium [RPMI 1640 with 10% human serum, penicillin (100 U/ml), streptomycin (100 µg/ml), fungizone (1.25 µg/ml) (Bristol-Myers Squibb), and interleukin-2 (IL-2) (6000 U/ml)] at 37°C and 5% CO2, allowing TILs to migrate into the medium. TILs were expanded to reach >50 × 10^6 total cells or confluent growth in 2-ml wells and eliminated adherent tumor cells (average of ~2 × 10^8 cells per well from each tumor fragment). TIL cultures were further expanded using a standard rapid expansion protocol (REP) as previously described (36). Briefly, TILs were stimulated with anti-CD3 antibody (30 ng/ml) (OKT-3; Ortho Biotech) and IL-2 (6000 U/ml) in the presence of irradiated (40 grays) allogenic feeder cells (healthy donor PBMCs) at a feeder/TIL ratio of 200:1. Initially, TILs were rapidly expanded in a 1:1 mix of complete medium and REP medium [AIM-V (Invitrogen) + 10% human serum, fungizone (1.25 µg/ml), and IL-2 (6000 U/ml)], but after 7 days, complete medium and serum were removed stepwise from the culture by adding REP medium without serum to maintain cell densities around 1 × 10^7 to 2 × 10^6 cells/ml. TIL cultures were cryopreserved at −150°C in human serum + 10% DMSO. Mouse spleen suspensions were obtained by mashing the full spleen through a 70-µm cell strainer (Thermo Fisher Scientific). Red blood cells (RBC) were lysed with RBC Lysis buffer (BioLegend) and used directly or cryopreserved at −150°C in FCS (Gibco) + 10% DMSO.

**Mutation analysis and neoantigen prediction**

DNA and RNA were extracted and purified from tumor fragments and PBMCs (germline DNA reference) using the AllPrep DNA/RNA Mini Kit (Qiagen, 80204), with the addition of deoxyribonucleoside during RNA purification (Qiagen, 79254). Hereafter, DNA/RNA concentrations were analyzed using NanoDrop (Thermo Fisher Scientific), and RNA RIN values were analyzed by 2100 Bioanalyzer (Agilent Technologies). DNA whole-exome and RNA sequencing were performed at the DTU Multi Assay Core. A total of 276 identified missense mutations from tumor fragments of melanoma patient MM909.37 were used to predict 9–11-mer HLA-restricted peptides and sorted according to Mut_MHCrank up to the score of 2 using MuPexi and netMHCPan-2.8 (18). A total of 43 A2-restricted neoepitopes were predicted.

**Statistical analysis**

All the statistical analyses were done using GraphPad Prism 7 data analysis software. Paired t tests were performed to compare staining indices shown in Fig. 2, C and F.

**SUPPLEMENTARY MATERIALS**

immunology.sciencemag.org/cgi/content/full/4/37/eaau9039/DC1

Fig. S1. MD simulation of DS-A2.

Fig. S2. Dipeptide-mediated folding efficiency of DS-MHC molecules.

Fig. S3. Peptide binding measurements and comparative purification profiles.

Fig. S4. Gating strategies for flow cytometry analysis.

Fig. S5. Purity and biotinylation efficiency.

Fig. S6. Detection of virus-derived antigen-specific T cells from donor PBMCs in a combinatorial encoding strategy.

Fig. S7. Detection of low-avidity pMHC-TCR interactions using DS–H-2Kb multimers.

Fig. S8. T cell detection plots for the stability analysis of DS-A2 empty-loadable tetramers.

Fig. S9. T cell detection plots of peptide dissociation analysis.

Table S1. Crystal structure data collection and refinement statistics.

Table S2. Protein yield after in vitro folding and purification of wt and DS-A2 molecules.

Table S3. Raw data (Excel file).

**REFERENCES AND NOTES**


Dipeptides catalyze rapid peptide exchange on *S.* *T.* HLA-A1, -A3, -A11, and -A2. *D.* Dipeptides promote folding and Peptide-independent stabilization of *N.* *S.* *P.* An analysis of natural T cell responses to *J.* *C.* *D.* *R.* *D.* *R.* *C.* *S.* *M.* *C.* *J.* *D.* *B.* *K.* *A.* 12 of 13.

The other authors declare that they have no competing interests. **Data and materials availability:** The crystal structure data shown in this study for CMV pp65-NLVPMAATV/DS-A2 have been deposited in the Protein Data Bank with an ID number 6Q3K. All other data needed to evaluate the conclusions in the paper are present in the paper or the Supplementary Materials.

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Empty peptide-receptive MHC class I molecules for efficient detection of antigen-specific T cells

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Empty MHC reagents improve T cell detection

Recombinant MHC molecules displaying single peptides in their peptide-binding cleft are valuable reagents for identifying T cells that bind specific peptide-MHC complexes. Two studies in this week's issue show that disulfide-stabilized (DS) class I MHC molecules offer a more efficient path to preparing large libraries of MHC-peptide reagents. Saini et al. developed DS versions of three class I MHC molecules and used a library of DS-HLA-A2-peptide multimers to rapidly screen T cells infiltrating human melanoma tumors for neoantigen reactivity. Moritz et al. prepared libraries of DS-HLA-A2-peptide complexes to screen an affinity-matured TCR for cross-reactivity with self-peptide-MHC complexes. Empty class I MHC molecules that are stable and easily loaded with peptide will facilitate the wider use of MHC-peptide reagents for T cell detection. See related Research Article by Moritz et al. in this issue.